

## Persistent Echovirus Infection of Mouse Cells Expressing the Viral Receptor VLA-2

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Mouse cells are not susceptible to infection with echovirus 1 (EV-1) because they lack the viral receptor, human VLA-2. Two mouse fibroblast cell lines, L cells and 3T3 cells, were made susceptible to EV-1 infection after transformation with cDNAs of human VLA-2. After EV-1 infection, L cell transformants of human VLA-2 ( $\alpha 2\beta 1$  L cells) develop cytopathic effect (CPE) as expected, while 3T3 cell transformants of human VLA-2 ( $\alpha 2\beta 1$  3T3 cells) or the  $\alpha 2$  subunit of human VLA-2 ( $\alpha 2$  3T3 cells) become persistently infected. The distinct outcome is not a result of differential virus growth on these transformants because one-step growth curve analysis reveals little difference in EV-1 replication in both cell lines. In addition, 3T3 cell transformants expressing the poliovirus receptor (Pvr 3T3 cells) are lysed during poliovirus infection, suggesting that 3T3 cells are not intrinsically resistant to CPE caused by enterovirus infection. The results of limit dilution assays indicate that all EV-1-infected  $\alpha 2$  3T3 cells produce infectious virus. All EV-1-infected  $\alpha 2$  3T3 cells remain viable after EV-1 infection, and the kinetics of cell growth were not altered. FACS analysis reveals that receptor down-regulation is not involved in the establishment of persistent infection. Furthermore, inhibition of host protein synthesis was not observed in EV-1-infected  $\alpha 2$  3T3 or  $\alpha 2\beta 1$  L cells. Since  $\alpha 2\beta 1$  L cells are lysed by EV-1 infection, these findings suggest that virus-induced translation inhibition is not a determinant of cell killing. © 1997 Academic Press

### INTRODUCTION

Polioviruses, echoviruses, and coxsackie viruses, members of the *Enterovirus* genus of the *Picornaviridae*, are small, nonenveloped, positive-stranded RNA viruses (Rueckert, 1996). Enteroviruses are the causative agents of various human diseases including paralytic poliomyelitis, aseptic meningitis, myocarditis, hepatitis, and acute hemorrhagic conjunctivitis (Melnick, 1996). Although the hallmark of enterovirus infection of cultured cells is the development of cytopathic effect (CPE), persistent infections, during which CPE fails to develop, have also been reported for poliovirus, coxsackievirus, and echovirus (Colbere-Garapin *et al.*, 1989; Gibson and Righthand, 1985; Klingel *et al.*, 1992; Lloyd and Bovee, 1993; Lopez-Guerrero *et al.*, 1989; Okada *et al.*, 1987; Pavio *et al.*, 1996). *In vivo* persistent enterovirus infections have been implicated in the etiology of several human diseases, including myocarditis (Klingel *et al.*, 1992), postpolio syndrome (Sharief *et al.*, 1991), amyotrophic lateral sclerosis—motor neuron disease (Swanson *et al.*, 1995), and chronic fatigue syndrome (Cunningham *et al.*, 1990).

Viral persistence is controlled by both viral and cellular determinants. Reduced viral lytic potential (Pelletier *et al.*, 1991) and the presence of defective virus particles (Gibson and Righthand, 1985) or subgenomic replicons

(Kaplan *et al.*, 1989) can lead to the establishment of persistent infection. The level of virus receptors on the cell surface and the differentiation state of the cell have been shown to be cellular determinants of viral persistence in cultured cells (Borzakian *et al.*, 1992; Kaplan and Racaniello, 1991). After persistent poliovirus infection of HeLa cells or HEp-2c cells, cell surface expression of the poliovirus receptor (Pvr) is downregulated. Consistent with the lower levels of Pvr expression, the selected cells display an increased resistance to superinfection. In addition, well differentiated human blood cell lines develop CPE more rapidly than less differentiated cell lines (Okada *et al.*, 1987), and after hemin-induced differentiation, poliovirus infection of K562 cells becomes cytolytic rather than persistent (Benton *et al.*, 1996).

The identification of cellular receptors for viruses enables the expression of receptors in cells that do not normally bind virus. The study of such cell lines is likely to reveal additional determinants of viral persistence. The poliovirus receptor (Pvr) has been identified as a novel member of the immunoglobulin protein superfamily (Mendelsohn *et al.*, 1989). The cell receptor for echovirus 1 (EV-1) and echovirus 8 (EV-8) is the human very late antigen-2 (VLA-2) (Bergelson *et al.*, 1992). These EV serotypes have been associated with aseptic meningitis, paralysis, exanthema, respiratory disease, epidemic myalgia, pericarditis, and myocarditis (Melnick, 1996). Human VLA-2 consists of two subunits: an  $\alpha 2$  subunit, which contains the binding site for EV-1 (King *et al.*, 1995), and a  $\beta 1$  subunit, which is required for cell surface expression of the  $\alpha 2$  subunit.

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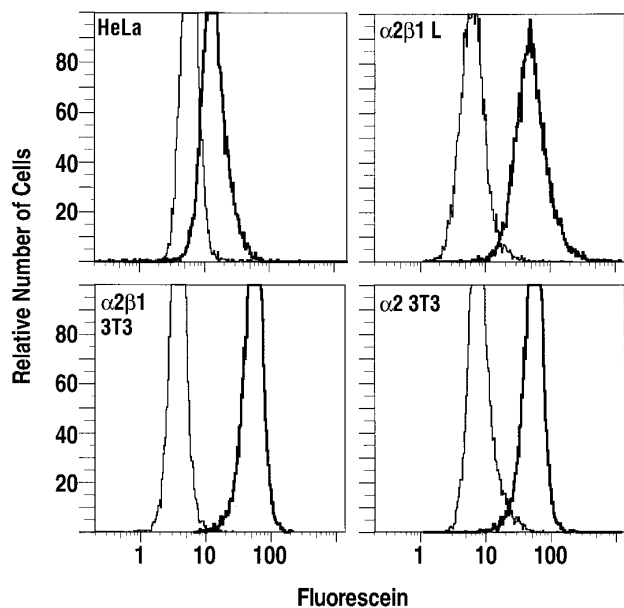


FIG. 1. Cell surface expression of the  $\alpha 2$  subunit of human VLA-2. HeLa cells,  $\alpha 2\beta 1$  L,  $\alpha 2\beta 1$  3T3, and  $\alpha 2$  3T3 cell transformants were stained with a mouse anti- $\alpha 2$  monoclonal antibody and then with a goat anti-mouse IgG antibody conjugated with fluorescein, followed by flow cytometry (thick lines). Cells that were not stained with the anti- $\alpha 2$  monoclonal antibody were used as negative controls (thin lines).

By expressing VLA-2 in mouse cells, we have identified a new cell culture model of EV persistence. Stable mouse L cell and 3T3 cell transformants that express human VLA-2 ( $\alpha 2\beta 1$  L and  $\alpha 2\beta 1$  3T3 cells) were isolated. Upon infection with EV-1 or EV-8, CPE develops on  $\alpha 2\beta 1$  L cells, but not on  $\alpha 2\beta 1$  3T3 cells, while the kinetics of virus growth on both cell lines are similar. However, 3T3 cell transformants expressing Pvr develop CPE after infection with poliovirus, indicating that 3T3 cells are not intrinsically resistant to CPE caused by enterovirus infection. No changes in cell surface levels of VLA-2 were detected after EV-1 infection of  $\alpha 2$  3T3 cells, indicating that receptor down-regulation is not involved in this persistent infection. Inhibition of host protein synthesis was not observed during echovirus infection of both cell lines, suggesting that virus-induced translation inhibition is not a determinant of cell killing. The 3T3 and L cell VLA-2 transformants are new systems for studying the mechanism of CPE development and persistent infection by echoviruses.

## MATERIALS AND METHODS

### Cells and viruses

HeLa S3 cells were grown in suspension cultures in Joklik minimal essential medium supplemented with 5% horse serum and gentamycin (10  $\mu\text{g/ml}$ ). For plaque assays, HeLa cells were plated in Dulbecco modified Eagle medium (DMEM) containing 5% bovine calf serum

and gentamycin. Mouse L cells and 3T3 cells were maintained in DMEM containing 10% bovine calf serum, penicillin (100 U/ml), and streptomycin (100  $\mu\text{g/ml}$ ). Stable transformants were maintained in the same medium containing G418 (Geneticin; Sigma) (200  $\mu\text{g/ml}$ ).

Poliovirus strain P1/Mahoney was derived from the infectious cDNA clone (Racaniello and Baltimore, 1982) and grown on HeLa cells. EV-1 (Farouk strain) and EV-8 (Bryson strain) were obtained from American Type Culture Collection and were grown on HeLa cells. Stocks of wild-type viruses were prepared after triple plaque purification. Stocks of mutant viruses from persistently infected  $\alpha 2$  3T3 cells were not plaque purified due to technical difficulties imposed by the small plaque phenotype.

### cDNAs

Poliovirus receptor cDNA was cloned previously (Mendelsohn *et al.*, 1989). cDNAs encoding human VLA-2 were cloned by PCR. For the 5'-half of the  $\alpha 2$  subunit, oligonucleotides 5'-CATGCTCTAGAGAATTCCTGCAAA-CCCAG-3' (sense; *Xba*I site underlined) and 5'-AGACAC-ATCGGTGATGGA-3' (antisense) were used as primers. For the 3'-half of the  $\alpha 2$  subunit, oligonucleotides 5'-ATCAGGGCACTATCCGCAC-3' (sense) and 5'-ATAGC-TCTAGACTGCAGGTAGGTCTGCTG-3' (antisense; *Xba*I site underlined) were used as primers. PCR products of the  $\alpha 2$  subunit 5'-half and 3'-half share a small stretch of DNA sequence that contains a unique *Bam*HI site. For the  $\beta 1$  subunit 5'-half, oligonucleotides 5'-ATAGCGG-ATCCAAGATGAATTTACAACCAAT-3' (sense; *Bam*HI site underlined) and 5'-CCAGCCAATCAGTGATCCAC-3' (antisense) were used as primers. For the  $\beta 1$  subunit 3'-half, oligonucleotides 5'-GTGAACAGAACTGCACCACC-3' (sense) and 5'-CATGCGGATCCCAGTGTGTGGGATT-TGCAC 3' (antisense; *Bam*HI site underlined) were used as primers. PCR products of the  $\beta 1$  subunit 5'-half and 3'-half share a small stretch of DNA sequence that contains a unique *Taq*I site. Genomic DNA of a human VLA-2 cDNA transformed Chinese hamster ovary (CHO) cell line was used as template (Bergelson *et al.*, 1993).

PCR products were cloned into mammalian cell expression vector pSVL (Pharmacia) by three-way ligation. Briefly, the ligation mixture contained vector pSVL cleaved with *Xba*I, the 5'-end of  $\alpha 2$  subunit cDNA cleaved with *Xba*I and *Bam*HI, and the 3'-half of  $\alpha 2$  subunit cDNA cleaved with *Bam*HI and *Xba*I.  $\alpha 2$  subunit encoded by the cloned cDNA was expressed on the cell surface of CHO cells to confirm its ability to bind virus (cell surface expression of the  $\alpha 2$  subunit in CHO cells can be achieved without human  $\beta 1$ , presumably due to the presence of endogenous  $\beta 1$ ). Receptor function of both  $\alpha 2$  and  $\beta 1$  subunit cDNAs was tested by transiently transforming these cDNAs into mouse L cells and assaying for growth of EV-1.

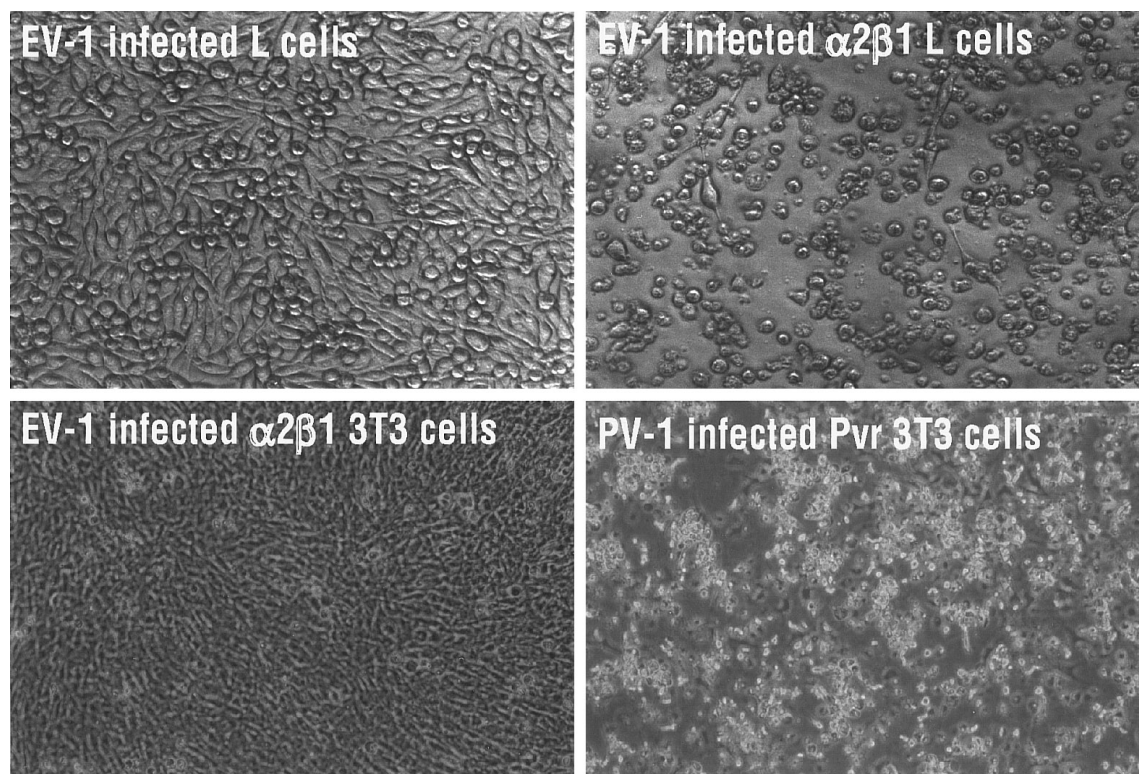


FIG. 2. Development of cytopathic effect (CPE) after virus infection. Cells were infected with echovirus 1 or poliovirus at an m.o.i. of 10 and photographed with a Nikon phase contrast microscope under visible light. Photographs were taken at 3 days postinfection except for Pvr 3T3 cells, which were photographed 2 days after infection with poliovirus. Magnification: L cells,  $\alpha 2\beta 1$  L cells, 200 $\times$ ;  $\alpha 2\beta 1$  3T3 cells and Pvr 3T3 cells, 100 $\times$ .

### DNA transformation

Mouse Ltk- cells and 3T3 cells were seeded in plastic cell culture plates 1 day before use at  $7.5 \times 10^5$  cells per 10-cm-diameter plate. Cells were treated with 1 ml of a DNA-calcium phosphate coprecipitate consisting of 10  $\mu$ g of cDNA and 1  $\mu$ g of plasmid pcDNA1neo (Invitrogen), which contains the neomycin resistance gene. After 20 hr of incubation at 37 $^\circ$ , the medium was replaced, and incubation continued for an additional 24 hr prior to the addition of geneticin. After 2 weeks of growth in the presence of geneticin (400  $\mu$ g/ml), geneticin-resistant colonies were either subcultured and characterized by fluorescence-activated cell sorting (FACS), or colonies were expanded together and cloned by FACS. Individual cells were then grown up and characterized by FACS. One clone from each transformation plate was chosen for further analysis.

### FACS staining and analysis

Anti-Pvr monoclonal antibody (mAb) 711C was generated as previously described (Morrison *et al.*, 1994). Anti-human  $\alpha 2$  mAb IOP49b, and anti-human  $\beta 1$  mAb IOT29 were obtained from AMAC, Inc. For analysis of human VLA-2 expression, cells were detached from tissue culture plates by treatment with trypsin-EDTA. Since Pvr is sensitive to trypsin treatment, for analysis of human PVR

expression, cells were detached by treatment with enzyme-free dissociation buffer (GIBCO). For analysis of receptor expression, living cells were stained in a volume of 50  $\mu$ l containing mAb, PBS, and 3% newborn calf serum. For FACS cloning, cells were stained in a volume of 200  $\mu$ l. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and IgM antibody (TAGO) was used as the secondary antibody. Samples prepared in this way were subjected to FACS analysis on a Becton-Dickinson FAC-Star with the laser excitation wavelength set at 488 nm. Propidium iodide was used to allow the exclusion of dead cells.

### Virus infection

To ensure adequate adhesion of 3T3 cells or 3T3 transformants to tissue culture plastic, 3 days before infection, approximately  $4 \times 10^5$  cells were seeded in a T25 tissue culture flask with one replacement of medium 12 hr before infection. For L cells or L cell transformants, cells were seeded 2 days before infection. All infections were carried out at a multiplicity of infection (m.o.i.) of 10 unless otherwise indicated. After a 45-min adsorption at 37 $^\circ$ , all flasks were washed with phosphate-buffered saline (PBS) three times to remove unbound virus before the addition of medium. For assaying virus growth, cell culture supernatant was taken at different times postin-

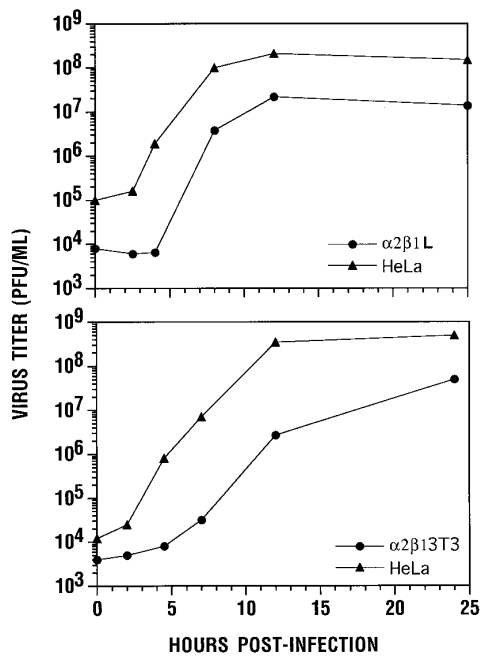


FIG. 3. One-step growth curve analysis of echovirus 1.  $\alpha 2\beta 1$  L and 3T3 cell transformants and HeLa cells were infected with echovirus 1 at an m.o.i. of 10. Virus titers in the supernatants taken at different times after infection were determined by plaque assay on HeLa cell monolayers.

fection and subjected to plaque assay on HeLa cells. For observation of CPE, infection was allowed to proceed at 37° and the medium was changed every 2 to 3 days.

Limiting dilution assay

Two hours after infection with EV-1 at an m.o.i. of 10,  $\alpha 2$  3T3 cells were treated with trypsin and washed exten-

sively to remove virions attached to the cell surface. A series of dilution of the cells was made, and the concentration of cells in the suspension was determined with a hemacytometer. One hundred microliters of each dilution were added to either HeLa cell monolayers grown on a 96-well plate to assay the release of plaque forming units (PFU) or to an empty well to observe cell growth. To subtract out the cells that died during the washes and cannot support virus replication, the ratio of PFU-releasing cells to the total number of cells was calculated by comparing the average number of cells needed to seed one well of a 96-well plate and that needed to induce CPE on HeLa cells cultured on a 96-well plate. To rule out that the virus detected in this assay was not simply adhering to the infected  $\alpha 2$  3T3 cells, virus was adsorbed to cells and after 1 hr incubation, the cells were frozen and thawed three times, and infectivity was determined by plaque assay on HeLa cell monolayers. The results indicate that on average, one infectious virus particle is associated with every five cells, compared to at least one infectious virus per cell as determined in the limit dilution assay.

Analysis of protein synthesis

Synthesis of protein in virus-infected cells was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of [<sup>35</sup>S]methionine-labeled cytoplasmic extracts. At different times after infection, the medium was removed and replaced with Dulbecco minimal essential medium with D-glucose, without methionine, and supplemented with 10  $\mu$ Ci [<sup>35</sup>S]methionine per milliliter. After 15 min of incubation at 37°, medium was removed and cells were washed with 1 ml of cold PBS. Cytoplasmic extracts were made by lysing cells in ice-

TABLE 1  
Percentage of 3T3 Cell Transformants of the  $\alpha 2$  Subunit of Human VLA-2 That Produce Infectious Particles after Echovirus 1 Infection

	Cell dilution				Cells required <sup>a</sup>	Infection ratio <sup>b</sup>
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>		
CPE <sup>c</sup>	12/12	10/12	2/12	0/12	1.6	100%
Cell <sup>d</sup>	12/12	9/12	3/12	0/12	1.6	
CPE	12/12	12/12	5/12	1/12	1.2	100%
Cell	12/12	12/12	6/12	0/12	1.2	

Note.  $\alpha 2$  3T3 cells were infected with echovirus 1 at an m.o.i. of 10, resuspended, and extensively washed to remove virions attached to the cells. 100  $\mu$ l of each dilution of cell suspension was added to a 96-well plate containing monolayers of HeLa cells.

<sup>a</sup> Average number of cells needed to seed one well or to induce CPE on HeLa cell monolayers was calculated to subtract out the cells that died during the washes.

<sup>b</sup> The infection ratio was calculated by dividing the average number of cells needed to seed one well by the number needed to induce CPE on HeLa cell monolayers and converting to percentage.

<sup>c</sup> Development of cytopathic effect (CPE) on the HeLa cell monolayer was considered a result of production of infectious particles by transferred 3T3 cells. Shown is the number of wells that developed CPE/total number of wells.

<sup>d</sup> Dilutions of cell suspension were added to empty wells; shown is the number of wells in which colonies of cells were observed/total number of wells.

cold PBS containing 0.5% Nonidet P-40 (NP40) followed by removal of nuclei by centrifugation. A portion of each extract was fractionated on a 12.5% SDS–polyacrylamide gel. After electrophoresis, gels were dried and exposed to X-ray film at room temperature.

## RESULTS

### Cytopathic effect develops on mouse L cell transformants ( $\alpha 2\beta 1$ L cells) but not on mouse 3T3 cell transformants of human VLA-2 ( $\alpha 2\beta 1$ 3T3 cells) after echovirus 1 infection

To determine whether echoviruses can replicate in cultured mouse cells, L cells and NIH 3T3 cells, two cell lines derived from mouse fibroblasts, were transformed with cDNAs that encode the  $\alpha 2$  and  $\beta 1$  subunits of human very late antigen 2 (VLA-2), the receptor for EV-1 and EV-8 (Bergelson *et al.*, 1992). Stable cell lines that express human VLA-2 were isolated by fluorescence-activated cell sorting (FACS) and named  $\alpha 2\beta 1$  L cells and  $\alpha 2\beta 1$  3T3 cells. FACS analysis demonstrated that the cell lines express human VLA-2 on the cell surface (Fig. 1). While surface expression of the  $\alpha 2$  subunit on L cells requires the presence of human  $\beta 1$  subunit (Zhang and Racaniello, unpublished results), transformation with  $\alpha 2$  cDNA alone is sufficient for surface expression on 3T3 cells (Fig. 1), presumably because of the presence of mouse  $\beta 1$  homolog in these cells. The difference between 3T3 and L cells may be a result of the loss of a few chromosomes in L cells (Kit *et al.*, 1963).

Two days postinfection with EV-1 at an m.o.i. of 10,  $\alpha 2\beta 1$  L cells developed obvious CPE (Zhang and Racaniello, unpublished results), and after 3 days all the cells had rounded up or detached from the plate (Fig. 2). No CPE was observed on EV-1-infected  $\alpha 2\beta 1$  3T3 cells at 3 days (Fig. 2) or 7 days postinfection (Zhang and Racaniello, unpublished results).  $\alpha 2$  3T3 cells resemble  $\alpha 2\beta 1$  3T3 cells in their resistance to EV-1-induced CPE (Zhang and Racaniello, unpublished results). Similar results were obtained using EV-8 (Zhang and Racaniello, unpublished results).

### Growth kinetics of EV-1 on $\alpha 2\beta 1$ L and $\alpha 2\beta 1$ 3T3 cells

To determine whether the distinct CPE phenotype was related to virus replication, growth kinetics of EV-1 on  $\alpha 2\beta 1$  L cells and  $\alpha 2\beta 1$  3T3 cells were determined. The results indicate that EV-1 replicates with similar kinetics, and to similar final levels on both murine cell lines (Fig. 3). Replication of EV-1 in HeLa cells was more rapid and produced higher final virus yields. At 24 hr postinfection, EV-1-infected  $\alpha 2\beta 1$  L cells and  $\alpha 2\beta 1$  3T3 cells produced 10 and 25 PFU/cell, respectively, while HeLa cells produced 1000 PFU/cell (Zhang and Racaniello, unpublished results). These results indicate that the absence of EV-

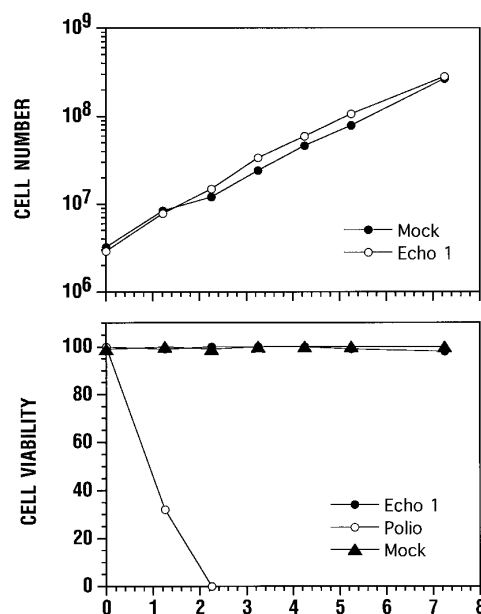


FIG. 4. Cell growth and cell viability of  $\alpha 2$  3T3 cells after echovirus 1 infection. Cells were infected with echovirus 1 at an m.o.i. of 10. Cell numbers were monitored daily with a hemacytometer, and cell viability was determined by trypan blue exclusion assay. PVR 3T3 cells infected with poliovirus were used as a control in the trypan blue exclusion assay.

1-induced CPE in  $\alpha 2\beta 1$  3T3 cells is not due to failure of the virus to multiply.

The growth kinetics of EV-1 in  $\alpha 2$  3T3 cells resembled that in  $\alpha 2\beta 1$  3T3 cells (Zhang and Racaniello, unpublished results). To avoid complications that might arise from the presence of two  $\beta 1$  subunits in 3T3 cells, subsequent experiments were carried out using  $\alpha 2$  3T3 cells.

To determine whether 3T3 cells are resistant to cell killing by another enterovirus, stable cell lines expressing Pvr were isolated by FACS, after transformation with PVR cDNA. Upon infection with poliovirus type 1, Pvr 3T3 cells develop CPE within 2 days (Fig. 2). These results suggest that mouse 3T3 cells are not intrinsically resistant to enterovirus-induced CPE.

### Most $\alpha 2$ 3T3 cells produce infectious virus after EV-1 infection

In many persistent virus infections of cultured cells, only a small fraction of the cells are infected (Joklik, 1977; Walker, 1964). A limiting dilution assay was therefore performed to determine the percentage of  $\alpha 2$  3T3 cells that produce infectious virus after EV-1 infection. The average number of cells required to cause CPE on HeLa cell monolayers was divided by the average number of cells required to form a colony, resulting in the fraction of live cells that produce infectious virus. The results indicate that 100% of  $\alpha 2$  3T3 cells produce infectious particles after high m.o.i. EV-1 infection (Table 1).

TABLE 2

Persistent Echovirus 1 Infection of  $\alpha 2$  3T3

Time postinfection	PFU/ml at hr postwash:	
	0	24
One month	$1.0 \times 10^5$	$7.9 \times 10^5$
Two month	$2.0 \times 10^3$	$4.7 \times 10^4$
Three month	$7.5 \times 10^2$	$1.5 \times 10^5$

*Note.*  $\alpha 2$  3T3 cell transformants were infected with echovirus 1 at an m.o.i. of 10, and at different times postinfection, the cells were washed and fresh medium was added. Samples of the supernatants were taken immediately or 24 hr after the wash, and virus titers were determined by plaque assay on HeLa cell monolayers.

$\alpha 2$  3T3 cells survive and divide after EV-1 infection

To ascertain whether EV-1-infected cells continue to grow, or if only a subpopulation of cells survives and divides, masking the death of other cells, the cell number and viability of EV-1-infected  $\alpha 2$  3T3 cells was determined. The results indicate that up to 7 days postinfection, EV-1-infected  $\alpha 2$  3T3 cells grow as well as their mock-infected counterparts (Fig. 4). In addition, viable cells account for more than 98% of the total number of cells, while the percentage of viable Pvr 3T3 cells dropped to zero within 2 days after poliovirus infection. These results indicate that most  $\alpha 2$  3T3 cells remain viable after EV-1 infection and continue to grow.

Continuous virus production by EV-1 infected  $\alpha 2$  3T3 cells

To determine whether EV-1-infected  $\alpha 2$  3T3 cells produce infectious virus for an extended period, cell culture supernatant was removed at 1 month, 2 months, and 3 months postinfection, cells were washed, and fresh medium was added. Virus titers in the cell culture supernatant were determined at zero and 24 hr after the wash. At all three time points, a significant increase in virus titer was observed at 24 hr compared to 0 hr (Table 2). These results indicate that  $\alpha 2$  3T3 cells continue to produce EV-1 up to 3 months after infection.

After 3 months of passage, persistently infected  $\alpha 2$  3T3 cells produced virus that forms pinpoint plaques on HeLa cells (Zhang and Racaniello, unpublished results), indicating the emergence of viral mutants. This small plaque phenotype was not observed in yields of earlier passages. Like previous observations of poliovirus persistent infections (Borzakian *et al.*, 1993), EV-1 mutants derived from two independently passaged persistent infections show a similar small plaque phenotype. These EV-1 mutants are temperature sensitive when assayed on HeLa cell monolayers (Table 3). Like wild-type EV-1, these mutants do not lyse fresh  $\alpha 2\beta 1$  3T3 cells at 32 or 37° (Zhang and Racaniello, unpublished results).

TABLE 3

Temperature Sensitivity of Echovirus 1 Isolated from Persistently Infected Cells

Virus	Virus titer (PFU/ml)		
	32°	37°	40°
wt EV1	$7.5 \times 10^{7b}$	$7.2 \times 10^7$	$2.8 \times 10^7$ (pinpoint)
pi EV1 <sup>a</sup>	$2.5 \times 10^4$ (pinpoint)	$2.2 \times 10^4$ (pinpoint)	<10

<sup>a</sup> Supernatant taken after 3 months of passage of  $\alpha 2$  3T3 cells persistently infected with echovirus 1.

<sup>b</sup> Virus titers (PFU/ml) were determined by plaque assay on HeLa cell monolayers. Plaque phenotype is shown in parenthesis.

Since it was previously reported that mutant viruses isolated from persistent poliovirus infection of human neuroblastoma cells have reduced ability to lyse a different cell line, we determined whether mutant EV-1 can lyse HeLa cells. Cells infected with wild-type EV-1 or the mutant EV-1 isolates develop CPE at similar rates (Zhang and Racaniello, unpublished results).

Persistent echovirus infection of  $\alpha 2$  3T3 cells is not caused by down-regulation of receptor expression

When persistent infections are caused by down-regulation of cell receptor expression, the persistently infected cells are usually resistant to superinfection. To address this question, we determined whether persistently infected  $\alpha 2$  3T3 cells can be superinfected with wt EV-1. The emergence of small plaque EV-1 variants during persistent infection made it possible to distinguish these viruses from the superinfecting wt virus. The results demonstrate that persistently infected  $\alpha 2$  3T3 cells can be superinfected with wt EV-1 (Table 4).

To confirm that down-regulation of the EV-1 receptor does not occur in persistently infected  $\alpha 2$  3T3 cells, sur-

TABLE 4

Superinfection of Persistently Infected  $\alpha 2$  3T3 Cell Transformants

Hour postinfection	Virus titer (PFU/ml)	
	Mock-infected cells	Super-infected cells
0	$5.0 \times 10^3$	$3.0 \times 10^4$
24	$3.0 \times 10^5$	$2.5 \times 10^7$
Plaque phenotype <sup>a</sup>	Pinpoint	wt

*Note.*  $\alpha 2$  3T3 transformants were infected with echovirus 1 and passaged for 3 months. Cells were then infected again with echovirus 1 or mock-infected. After infection, cells were washed and samples of the supernatant taken 0 and 24 hr later. Virus titers were determined by plaque assay on HeLa cell monolayers.

<sup>a</sup> Determined on HeLa cell monolayers.

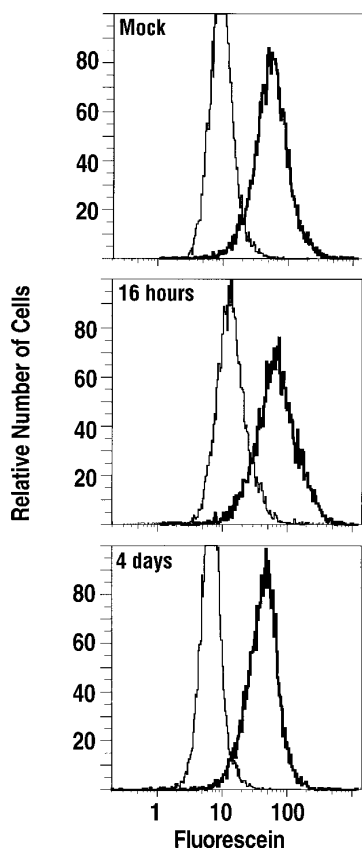


FIG. 5. Receptor expression on  $\alpha 2$  3T3 cells after echovirus 1 infection. Cells were infected with echovirus 1 at an m.o.i. of 10. At different times after infection,  $\alpha 2$  3T3 cells were fixed, stained with a mouse anti- $\alpha 2$  monoclonal antibody, then with goat anti-mouse IgG conjugated with fluorescein, followed by flow cytometry (thick lines). Cells that were not stained with the anti- $\alpha 2$  monoclonal antibody were used as negative controls (thin lines).

face levels of the  $\alpha 2$  subunit were determined by FACS analysis at different times after EV-1 infection of  $\alpha 2$  3T3 cells. No significant changes in  $\alpha 2$  levels on the cell surface were observed at 16 hr or 4 days after high m.o.i. EV-1 infection (Fig. 5). Therefore, receptor down-regulation is not responsible for the resistance of  $\alpha 2$  3T3 cells to EV-1-induced CPE.

#### Virus-induced shutoff of host translation does not occur in $\alpha 2$ 3T3 or $\alpha 2\beta 1$ L cell transformants

The inhibition of host protein synthesis observed in cells infected with members of the *Picornaviridae* is believed to play an important role in cell killing. Persistent poliovirus infection of human K562 cells correlates with failure to inhibit cell translation (Benton *et al.*, 1995). We therefore examined whether host protein synthesis is inhibited upon EV-1 infection of  $\alpha 2$  3T3 cells. Host translational inhibition was observed in HeLa cells infected with EV-1 (Fig. 6), although not as efficiently as the shutoff observed in HeLa cells infected with poliovirus (Zhang and Racaniello, unpublished results). However, shutoff

of host protein synthesis was not observed in EV-1-infected  $\alpha 2$  3T3 cells or  $\alpha 2\beta 1$  L cells (Fig. 6). These results were unexpected since EV-1 infection of  $\alpha 2\beta 1$  L cells leads to cytopathic effect (Fig. 1).

## DISCUSSION

Two different mouse cell lines have been isolated that express the cell receptor for echovirus 1,  $\alpha 2\beta 1$ . After infection with echovirus 1, L cells that express  $\alpha 2\beta 1$  develop CPE. In contrast, 3T3 cells expressing  $\alpha 2\beta 1$  do not develop CPE and become persistently infected. Poliovirus infection of either cell line expressing Pvr leads to cell killing. These cell lines provide a means of studying the mechanisms of echovirus-induced cell killing and establishment of persistent infections. Previously, WISH cell lines that are persistently infected with echovirus were isolated from the small population that survived lytic infection with echovirus 6 (Gibson and Righthand, 1985). Echovirus 6 infection of WISH cells results in killing of the majority of the cells, and the surviving cells are likely to be mutants that support persistent infection. In addition, the viruses produced during persistent infection are unable to attach to susceptible cells (Righthand and Blackburn, 1989). This persistent infection therefore differs considerably from those we have established in  $\alpha 2\beta 1$ -expressing 3T3 cells.

Why does infection with echovirus 1 result in cell killing in  $\alpha 2\beta 1$  transformants of L cells but not 3T3 cells? The results of one-step growth curve analyses indicate that echovirus 1 replicates in  $\alpha 2\beta 1$  3T3 cells as efficiently as in  $\alpha 2\beta 1$  L cells. Although the yield of EV-1 in  $\alpha 2\beta 1$  L cells is initially more rapid than in  $\alpha 2\beta 1$  3T3 cells, viral growth in  $\alpha 2\beta 1$  3T3 cells eventually surpasses that in  $\alpha 2\beta 1$  L cells. Therefore, it is unlikely that resistance of  $\alpha 2\beta 1$  3T3 cells to viral CPE is a result of poor viral growth. In addition, the CPE resistance of  $\alpha 2\beta 1$  3T3 cells cannot be explained by infection of only a small fraction of cells, because our results indicate that 100% of  $\alpha 2\beta 1$  3T3 cells are initially infected. It was previously suggested that poliovirus CPE might be the result of Pvr-mediated signal transduction (Morrison *et al.*, 1994). In addition, it was shown that HIV-1 envelope protein gp120 can activate protein kinase C in astrocytes, inducing astrocytosis (Wyss-Corey *et al.*, 1996). Engagement of integrins triggers the phosphorylation of various tyrosine kinases. Cross-linking of  $\beta 1$  integrins, in particular, leads to the phosphorylation of the protein kinases FAK and Syk (Clark and Brugge, 1995). It is possible that a necessary signal induced by virus occupancy of the echovirus 1 receptor, which normally is involved in cell killing, is abrogated in  $\alpha 2\beta 1$  and  $\alpha 2$  3T3 cells. Perhaps upon virus binding to the  $\alpha 2$  subunit in the  $\alpha 2\beta 1$  3T3 cells or  $\alpha 2$  3T3 cells, conformational changes cannot be induced in the mouse  $\beta 1$  subunit because of the differences between human  $\alpha 2$  and its mouse homolog, leading to the

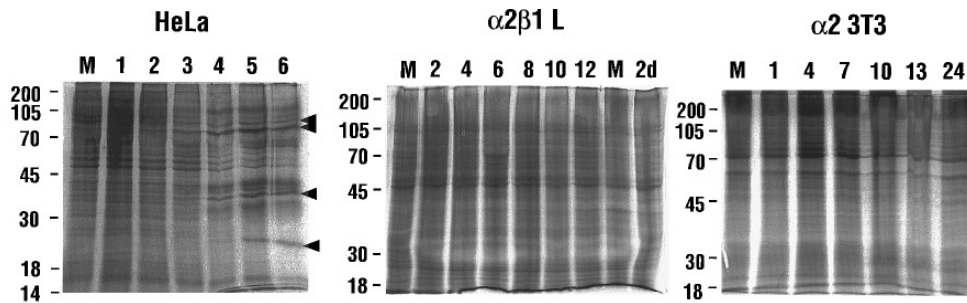


FIG. 6. Inhibition of host protein synthesis during virus infection. HeLa,  $\alpha 2\beta 1$  L, or  $\alpha 2$  3T3 cells were infected with echovirus 1 at an m.o.i. of 100. At different times after infection, cells were labeled with [ $^{35}$ S]methionine for 15 min. Cytoplasmic lysates were prepared and fractionated by SDS-PAGE. Gels were dried and exposed to X-ray film. M, mock-infected cells; numbers above lanes represent hours postinfection, except 2d (2 days postinfection). Positions of molecular weight markers are shown at the left of each gel; sizes are given in kDa. Arrowheads indicate several of the echovirus 1 proteins whose synthesis increase during infection of HeLa cells. No echovirus proteins could be detected in infected  $\alpha 2\beta 1$  L or  $\alpha 2$  3T3 cells over the background of cell proteins. However, these cells were successfully infected as judged by the production of virus in the culture supernatants (Zhang and Racaniello, unpublished results).

absence of signal transduction by the mouse  $\beta 1$  subunit. Alternatively, signal transduction in  $\alpha 2\beta 1$  3T3 cells or  $\alpha 2$  3T3 cells might prevent CPE formation, while abrogation of signaling in L cell transformants might allow CPE to develop. Another possible explanation for the differential susceptibility to EV-1-induced CPE might be related to changes in cell membrane permeability that occur during virus infection. Picornavirus infection of cells may enhance membrane permeability and release of lysosomal hydrolases into the cytoplasm, resulting in cytopathic effect (Carrasco, 1995; Wolff and Bubel, 1964). For example, lysosomal hydrolases in L cells might be more readily released by viral proteins than in 3T3 cells.

Results obtained from persistent poliovirus infection of human neuroblastoma cells suggest that the selection pressure during persistent infection tends to make the virus mutate in a fixed pattern (Borzakian *et al.*, 1993). The same missense and silent mutations accumulated in the genomes of viruses isolated from three independent persistent infections. Our results are consistent with this suggestion. Two independently passaged, persistently infected  $\alpha 2$  3T3 cells yielded mutant viruses that display identical phenotypes: a small plaque phenotype at 37° and temperature sensitivity. However, unlike previous observations that mutated viruses can persistently infect uninfected, susceptible cells (Borzakian *et al.*, 1993), the echovirus mutants isolated here retain their ability to lyse HeLa cells.

Inhibition of host protein synthesis by echovirus 22 is not efficient (Coller *et al.*, 1990). Our results suggest a similar property of echovirus 1 infection of HeLa cells. Furthermore, host protein synthesis shutoff does not occur during lytic echovirus 1 infection of  $\alpha 2\beta 1$  L cells. The absence of host translational inhibition during lytic echovirus infection is intriguing. Inhibition of host protein synthesis is believed to play an important role in cell killing, and the absence of translational shutoff was shown to correlate with the ability of poliovirus to persist

in human K562 cells (Lloyd and Bovee, 1993). Further studies will be required to determine whether virus-induced inhibition of host protein synthesis plays a role in cell killing by enteroviruses or is simply a by-product of virus replication.

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